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Early increased renal procollagen α1(IV) mRNA levels in streptozotocin induced diabetes

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Early increased renal procollagen α1(IV) mRNA levels in streptozotocin induced diabetes. Changes in renal procollagen mRNA levels were measured shortly after the induction of streptozotocin induced diabetes in the rat. "Medullary" procollagen α1(IV) levels seven days after diabetes induction was significantly higher in untreated diabetic rats (DM, $N = 12$; 244 ± 57% of the mean control value), than in diabetic rats receiving small doses of insulin insufficient to achieve euglycemia (NPH, $N = 10$; 87 ± 12%) and in diluent injected nondiabetic control rats (C, $N = 15$; 100 ± 12%; $P < 0.01$, DM vs. C and DM vs. NPH). "Medullary" procollagen α1(I) mRNA levels were numerically increased in DM to a lesser degree (141 ± 5%, ANOVA not significant) compared to C (100 ± 13%), and this small increment was further normalized by insulin treatment (NPH, 120 ± 11%). A trend for increased β-actin mRNA levels in DM did not reach significance ($P > 0.05$). Increases in "medullary" procollagen mRNA levels did not correlate with kidney weight, glomerular tuft volume, creatinine clearance, food intake, or body weight gain, and occurred when renal morphology was normal by light microscopy. Statistically significant but weak correlations were noted between the serum glucose levels and "medullary" procollagen α1(IV) mRNA levels ($r = 0.43$, $P < 0.05$). In addition, weak correlations were noted between glycosuria and "medullary" procollagen α1(I) levels ($r = 0.38$, $P < 0.05$). In situ hybridization studies localized the increased procollagen α1(IV) mRNA levels predominantly in the DM group primarily in the deep cortex and medullary outer stripe of proximal tubules. Glomerular procollagen α1(IV), α1(I), α1(III) and β-actin mRNA levels were not increased in untreated diabetic rats 7 or 28 days after diabetes induction. Thus, tubular procollagen α1(IV) mRNA levels increased prior to any measurable change in glomerular levels and were ameliorated by insulin administration.

Diabetic kidney disease is characterized by changes in both glomerular and tubular structure and function. Most studies have focused on alterations in the glomerulus, including abnormalities in glomerular permeability and capillary pressure [1–6], glomerular hyperplasia or hypertrophy [7–9], increases in basement membrane width and fractional increases in mesangial matrix volume [9–11]. Although the effects of diabetes on the renal tubulointerstitium have received relatively little attention, tubular lesions were described in diabetes more than a century ago [12, 13]. In fact, diabetic renal hypertrophy is largely a tubular process [14, 15], and abnormalities in tubular transport and intracellular composition have been reported [16–19], as have tubular basement membrane thickening and tubulointerstitial fibrosis [20–22].

Type IV collagen is a normal constituent of both tubular and glomerular basement membranes and mesangial matrix. As such, many experiments have focused on studying collagen synthesis and degradation as a means of understanding the pathogenesis of diabetic kidney disease [23–30]. In the present experiments, we tested the hypothesis that diabetic kidney disease is at least in part due to an abnormality in extracellular matrix gene expression. In these studies, in vitro and in situ hybridization techniques were used to measure and localize procollagen mRNA levels in the initial days after rats were rendered diabetic by streptozotocin. These studies demonstrate a prominent increase in procollagen α1(IV) mRNA levels localizing in proximal tubules of the deep cortex and outer stripe of the renal medulla which could be completely abrogated by low dose insulin insufficient to normalize renal size or serum glucose levels. These tubular changes preceded any measurable changes in glomerular procollagen mRNA levels.

Methods

Experimental animals

Seven-day in vitro hybridization studies. Male Sprague-Dawley rats intended for glomerular and/or medullary mRNA measurements were injected with streptozotocin 65 mg/kg i.p. in sodium citrate buffer, pH4 or with buffer alone ($N = 15$) and sacrificed after seven days. Induction of diabetes was ascertained by measuring glycosuria by dipstick 24 hours after streptozotocin administration. Ten of the diabetic rats received 2 units of NPH insulin s.c. daily (NPH) and twelve were left untreated (DM). The following physiological measurements were made prior to injection and again just before sacrifice seven days later: body weight; serum and urine glucose (YSI autoanalyzer II, Irvine, California, USA); hematocrit; systolic arterial blood pressure by the tail cuff method in awake restrained animals [31]; and 24-hour urine protein by sulfosalicylic acid precipitation and turbidometric analysis [32]. The food intake of each rat was measured daily beginning on the day of buffer or streptozotocin injection.

Twenty-eight day in vitro hybridization studies. Since initial in vitro and in situ hybridization studies revealed no significant

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changes in glomerular procollagen gene expression after only seven days, a later time point during the course of the disease process was deemed important to examine in untreated rats with diabetes and in controls.

Therefore an additional 14 rats underwent diluent ($N = 7$) or streptozotocin ($N = 7$) injection and were sacrificed after 28 days. Physiological measurements identical to those performed on rats sacrificed after seven days were made on these rats prior to sacrifice. Glomeruli of these rats were utilized to assess procollagen α1(IV) and β-actin mRNA levels 28 days after injection with streptozotocin or diluent.

In situ hybridization. Nine additional Sprague-Dawley rats administered streptozotocin or diluent were sacrificed either seven days or more than 120 days post-injection. Their kidneys were harvested solely for in situ hybridization studies.

Rats were anesthesized with ketamine 87 mg/kg and xylazine 13 mg/kg i.m. prior to sacrifice and euthanized by exsanguination under general anesthesia.

Renal histology

Thin coronal renal sections were fixed in alcoholic Bouin's solution, transferred after 24 hours into 70% ethanol, embedded in paraffin, sectioned, and stained with periodic acid Schiff reagent. Glomerular area was measured morphometrically in at least 50 glomeruli from each rat using a Zeiss light microscope and digitizer tablet. Glomerular tuft volume (GTV) was calculated using the following formula: GTV = 1.25 (glomerular area)$^{3/2}$ [32–34]. In vitro fixation, alcohol dehydration and paraffin embedding may result in smaller glomerular area than is measured in renal tissue fixed in situ [34].

In vitro hybridization studies—"Medulla"

Kidneys were removed from experimental and control rats and the cortex separated from the medulla manually. The latter tissue, consisting of a small segment of deep renal cortex (approximately 1 mm) including deep juxtamedullary glomeruli, and the renal medulla and papillae, are referred to as "medulla." "Medulla" samples were snap-frozen in liquid nitrogen. Total RNA was extracted either by the guanidinium thiocyanate (GT)/cesium chloride ultracentrifugation method [35] or by the GT/phenol chloroform extraction method of Chomczynski and Sacchi [36]. RNA was quantitated spectrophotometrically by optical density readings at 260 nm. The quality of the RNA was assessed by two methods. First, the optical density ratios of the extracted RNAs were measured at 260 nm and 280 nm. Secondly, formaldehyde agarose gel electrophoreses were performed, the samples stained with ethidium bromide, and transilluminated by ultraviolet light to ensure the presence of intact 28S and 18S RNA bands.

For slot blot quantitation, five serial dilutions of samples of total RNA from each rat were placed on nitrocellulose filters (Hybond C extra, Amersham International, Amersham, UK or Nitroplus 2000, Micron Separations, Inc., Westboro, Massachusetts, USA), vacuum baked at 80°C for two hours, prehybridized and subsequently hybridized overnight with specific ^{32}P radiolabeled cDNA probes for procollagen α1(IV), procollagen α1(I) or β-actin. (Characteristics of cDNA probes are detailed below). Procollagen α1(I) mRNA was measured because it translates for a collagen peptide chain of major importance in the development of tubulointerstitial fibrosis. β-actin mRNA was measured as a control. Hybridized filters were washed to a stringency of 0.2 × SSC at 62°C (Hybond C) or 0.1 × SSC (with 0.1% SDS for β-actin) at 37°C (Nitroplus 2000) and developed with Kodak XAR film with two Quanta III intensifying screens (Kodak, Rochester, New York, USA) at −70°C. Developed autoradiograms were quantitated by laser densitometry (Helena Laboratories, Beaumont, Texas, USA). Specific procollagen and β-actin mRNA levels were initially expressed in optical density units per microgram of total RNA as calculated based on linear regressions for each experimental animal. Values for each experimental rat were then expressed as the percent of the mean value obtained in control rats. Due to the use of two types of nitrocellulose paper, samples were directly compared only among control and experimental samples analyzed using the identical nitrocellulose paper type (Hybond C extra vs. Nitroplus 2000). In the final analysis, samples from each rat were included if: 1) 260/280 nm RNA optical density ratios were between 1.7 and 2.2; 2) formaldehyde agarose gel electrophoresis disclosed intact 28S and 18S ribosomal RNA bands; 3) a sufficient quantity of RNA was isolated to blot serial dilutions for slot-blot quantitation; and 4) the correlation coefficient for the linear regression of the slot-blot autoradiogram was ≥0.85 utilizing a minimum of three dilutions. The number of samples which met these criteria for each quantitative mRNA measurement of "medullary" tissue derived from rats with diabetes for seven days is in Table 1.

Table 1. Number of samples of "medullary" tissue from rats with diabetes for seven days

	C	NPH	DM
Procollagen α1(IV)	14	9	9
Procollagen α1(I)	9	9	6
β-actin	14	9	9

In vitro hybridization studies—Glomeruli

Glomeruli collected from rats 7 and 28 days post-injection were isolated from the renal cortex by differential sieving over three sieves of mesh diameters 75, 106 and 125 microns [37]. The resultant glomerular samples were ≥85% pure. For glomeruli, the same criteria were utilized for RNA use as for "medullary" tissue except that agarose gel electrophoreses were performed only on representative samples due to the small amount of RNA available from the glomerular pellet of each experimental animal. The number of glomerular samples available for analysis from control and experimental rats 7 and 28 days after injection are in Table 2.

cDNA probes

The following cDNA probes were utilized: 1) mouse procollagen α1(IV) cDNA pPE123 [38]; 2) human procollagen α1(I) cDNA Hf-677 [39]; 3) human procollagen α1(III) cDNA Hf-934 [40]; and 4) rat β-actin cDNA LK#280 [41]. Northern analysis demonstrated unique binding of each of the probes with rat mRNA. For pPE123 (Fig. 1A) and Hf-677 [42], this resulted in binding to mRNA of two molecular weights of 6.8 and 5.9 Kb for the former and 5.8 and 4.8 Kb for the latter. For LK#280, binding occurred to an mRNA of unique molecular weight (Fig. 1B), as was the case for Hf-934 (data not shown).

Table 2. Number of glomerular samples from control and experimental rats

	C		NPH	DM	
	7 days	28 days	7 days	7 days	28 days
Procollagen α1(IV)	6	7	5	3	7
Procollagen α1(I)	9	—	10	8	—
Procollagen α1(III)	8	—	6	9	—
β-actin	5	7	8	3	7

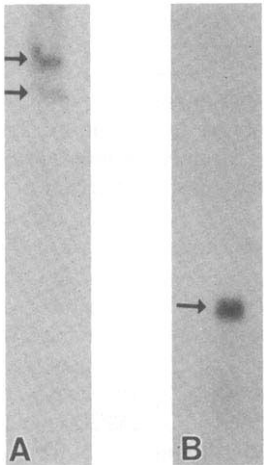


Fig. 1. *Northern electrophoretic gel analysis.* **A.** Binding of the procollagen α1(IV) cDNA probe pPE123 to mRNA bands of 6.8 and 5.9 kb (arrows). **B.** Binding of the β-actin cDNA probe LK#280 to a 1.2 kb mRNA band (arrows).

In situ hybridization

Quantitation of specific procollagen mRNA levels was ascertained by slot-blot analysis. However, identification of the specific cell types responsible for increased procollagen α1(IV) mRNA levels in the heterogeneous "medullary" tissue could not be ascertained by this method. Thus, for localization purposes only, nine additional animals were raised exclusively for in situ hybridization studies and sacrificed either seven days, or more than 120 days after diluent or streptozotocin injection. In situ hybridization was performed with procollagen α1(IV) cDNA pPE123, as well as with anti-sense and sense cRNAs subcloned from pPE123. Details of the subcloning procedure are described below. Hybridization was performed as described by Salido et al [43] with minor modifications.

Renal tissue was perfusion fixed via an abdominal aortic catheter with 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, followed by immersion in this fluid for 30 minutes. Fixed tissue was rinsed in PBS, cryoprotected in 15% sucrose in PBS for 30 minutes, embedded in OCT compound (Miles Laboratories, Elkhart, Indiana, USA), snap frozen in liquid nitrogen, sectioned at 6 to 10 microns, and mounted on glass slides coated with 50 μg/ml polylysine (ICN Immunobiologicals Irvine, California, USA). Sections were predigested with proteinase K, rinsed, and prehybridized for one to two hours at 50°C in 20 μl of a solution of 50% formamide, 0.3 M NaCl, 20 mM Tris (hydroxymethyl)-aminomethane (Tris) (pH 7.4), 5 mM EDTA, 100 mM dithiothreitol, 100 μg/ml salmon sperm DNA, and 10% dextran sulfate. As an additional negative control, some sections were also pre-incubated with RNase 50 μg/ml, 500 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA and 10 mM thiosulfate for 30 minutes at 37°C. Hybridization proceeded with the addition of the ^{35}S-labeled procollagen α1(IV) cDNA, control sense cRNA, or anti-sense cRNA probe to a solution identical to the prehybridization mixture. Siliconized cover slips were placed on the sections with the hybridization mixture and the hybridization reaction was carried out at 50°C for 24 hours. Cover slips were removed with 4 × SSC and the tissue was treated with 50 μg/ml RNase A for 30 minutes at 37°C and then washed for one hour in 2 × SSC at room temperature followed by 30 minutes in 0.1 × SSC at 50°C. Sections were dehydrated in a graded series of alcohol solutions, vacuum dried, dipped in a 50% aqueous solution of photographic emulsion NTB-2 (Eastman Kodak) at 45°C and exposed at 4°C for periods from 10 to 13 days in bakelite boxes with desiccant. Sections were developed with D19 developer (Eastman Kodak) and fixed with Rapid Fix (Eastman Kodak). Selected sections were counterstained with 0.5% methyl green. In addition, some sections were stained with periodic acid Schiff stain prior to the hybridization step. Sections were examined and photographed with a Zeiss Axiophot photomicroscope using bright field, dark field, and differential interference contrast condensers. The pathologist was blinded to the experimental group origin of the sections examined.

Preparations of cRNA probes

For generating procollagen α1(IV) sense and antisense RNA probes, the transcription vector pGEM-4Z (Promega Corporation, Madison, Wisconsin, USA) was used. Briefly, pPE123 cDNA type IV collagen-specific mouse clone was digested with EcoR I and Hind III and subcloned into appropriate sites within the multiple cloning site of pGEM-4Z. The resulting recombinant plasmid, pGEM-PE123, was then linearized with an appropriate restriction enzyme and in vitro transcription reactions were performed according to the recommended conditions with minor modifications. Transcription of 0.4 μg template DNA was carried out in a 30 μl reaction containing 10 mM DTT, 20 μmol each ot ATP, GTP and UTP, 20 units of RNasin and 20 units of SP6 or T7 RNA polymerase. Incubation was performed in the presence of ^{35}S-CTP (Amersham, specific activity 850 Ci/mmol) at 37°C for T7 and at 40°C for SP6 RNA polymerases. The DNA template was digested with RQ1 RNase-free DNase, the labelled cRNA was extracted with phenol/chloroform and chloroform and precipitated with ethanol. The specific activity of the probes obtained ranged from 2.0 to 4.0 × 10^8 cpm per microgram of RNA synthesized. The specificity of the probe was determined by using ^{32}P-labeled sense and anti-sense strands on Northern blots.

Statistics

Results are expressed as the mean ± standard error of the mean (SEM). Comparisons among groups were made by ANOVA using the Newman-Keuls multiple comparison test to further identify individual differences. Comparisons between groups were made using Student's *t*-test for normally distributed data and the Mann-Whitney rank sum test for non-parametric data. Significance was assigned at the $P < 0.05$ level.

Table 3. Physiological parameters in control (C), NPH insulin treated (NPH) and untreated diabetic (DM) rats just prior to sacrifice 7 days after diluent or streptozotocin injection

	C	NPH	DM
Serum glucose *mg/dl*	138 ± 8	284 ± 27[d,e]	446 ± 33[e]
Urine glucose *g/24 hr*	0.02 ± 0.01	1.00 ± 0.20[d,f]	3.90 ± 0.60[e]
Blood pressure *mm Hg*	114 ± 10	112 ± 5	114 ± 7
Creatinine clearance/100 g body wt	1.2 ± 0.2	1.7 ± 0.3[b]	0.9 ± 0.1
Hematocrit %	45 ± 1	46 ± 1	49 ± 1
Weight gain *% of initial weight*	27.8 ± 1.9	15.8 ± 0.9[b,e]	7.4 ± 3.5[e]
Food intake *g/24 hr/100 g body wt*	12.4 ± 0.4	15.0 ± 0.6[a]	15.4 ± 0.8[a]
Proteinuria *mg/24 hr*	7.3 ± 1.2	8.3 ± 1.8[f]	49.9 ± 10.23[e]
Kidney weight *g/2 kidneys*	1.7 ± 0.1	2.1 ± 0.1[b,c]	1.9 ± 0.1
Kidney weight/(body weight × 10^{-3})	9.0 ± 0.3	10.8 ± 0.3[c]	12.0 ± 0.5[c]

[a] $P < 0.05$ vs. C
[b] $P < 0.05$ vs. DM
[c] $P < 0.01$ vs. C
[d] $P < 0.01$ vs. DM
[e] $P < 0.001$ vs. C
[f] $P < 0.001$ vs. DM

Table 4. Physiological parameters in control and untreated diabetic rats just prior to sacrifice 28 days after diluent or streptozotocin injection

	C	DM
Serum glucose *mg/dl*	127 ± 5	448 ± 49[c]
Urine glucose *g/24 hr*	0.01 ± 0.00	4.89 ± 1.28[c]
Blood pressure *mm Hg*	145 ± 6	146 ± 7
Creatinine clearance/100 g body wt	2.2 ± 0.3	2.4 ± 0.5
Hematocrit %	50 ± 1	53 ± 1
Weight gain *% of initial weight*	44 ± 1	35 ± 1[b]
Food intake *g/24 hr/100 g body wt*	29.1 ± 0.6	43.3 ± 2.2[c]
Proteinuria *mg/24 hr*	17.4 ± 2.5	15.0 ± 0.5
Kidney weight *g/2 kidneys*	2.9 ± 0.1	3.6 ± 0.2[b]
Kidney weight/(body wt × 10^{-3})	8.6 ± 0.3	12.7 ± 0.6[c]

[a] $P < 0.05$
[b] $P < 0.01$
[c] $P < 0.001$

Results

Experimental animals

Mean values from rats sacrificed seven days after injection for serum and urine glucose, blood pressure, creatinine clearance, hematocrit, body weight, food intake, proteinuria, kidney weight, kidney weight to body weight ratio, and glomerular tuft volumes are summarized in Table 3.

Serum and urine glucose levels were highest in untreated diabetic rats (DM), intermediate in insulin treated diabetic rats (NPH) and physiologic in control rats (C) seven days after streptozotocin or buffer i.p. injection. Food intake/body weight was higher in DM and NPH compared to C, but weight gain was lower in DM and NPH compared with C. Body weights of all three groups were similar on initiation (DM = 156 ± 4 g; NPH = 171 ± 4 g; C = 155 ± 4 g), but significantly lower in the DM group at the termination of the experiment (DM = 167 ± 7 g; NPH = 199 ± 5 g; C = 197 ± 7 g; $P < 0.05$ DM vs. NPH and C). Creatinine clearance/body wt was highest in NPH, intermediate in C, and lowest in DM. Proteinuria was higher in DM than NPH and C. No differences were noted in systolic arterial blood pressure, serum creatinine, and hematocrit levels ($P > 0.05$). At sacrifice, absolute wet kidney weight was significantly increased only in the NPH group (C = 1.8 ± 0.1 g; NPH = 2.1 ± 0.1 g; DM = 1.9 ± 0.1 g; $P < 0.05$ vs. C and DM), but kidney weight corrected for BW was significantly greater in DM and NPH compared to C.

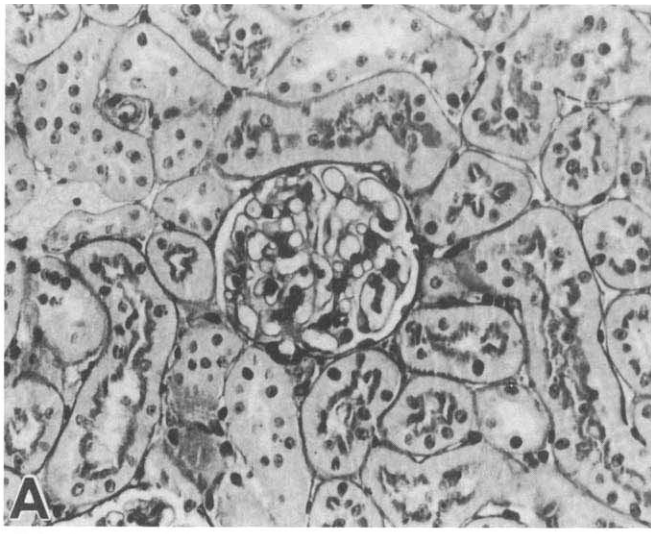
Mean values of physiologic measurements for diabetic and control rats sacrificed 28 days after injection are summarized in Table 4. Mean serum and urine glucose levels were high in untreated diabetic rats and physiologic in controls ($P < 0.001$). Food intake factored for body weight was more discrepant between the two groups at 28 days than was the case at 7 days, with diabetic rats eating approximately 50% more than controls. Despite the increased food intake, weight gain was 35 ± 1% of the initial body weight in the diabetic rats, and 44 ± 1% of the initial body weight in control rats ($P < 0.01$). Body weights were identical in the two groups prior to injection (C and DM 185 ± 3 g). At sacrifice C rats weighed 331 ± 7 g and DM weighed 285 ± 10 g ($P < 0.01$). Creatinine clearance corrected for body weight, proteinuria, systolic arterial blood pressure and hematocrit were not significantly different between the two groups ($P > 0.05$). At sacrifice, absolute kidney weight (C = 2.9 ± 0.1 g, DM = 3.6 ± 0.2 g, $P < 0.01$) and kidney weight corrected for body weight ($P < 0.001$) were higher in the diabetic than control rats, and these differences were greater than those present seven days after disease induction.

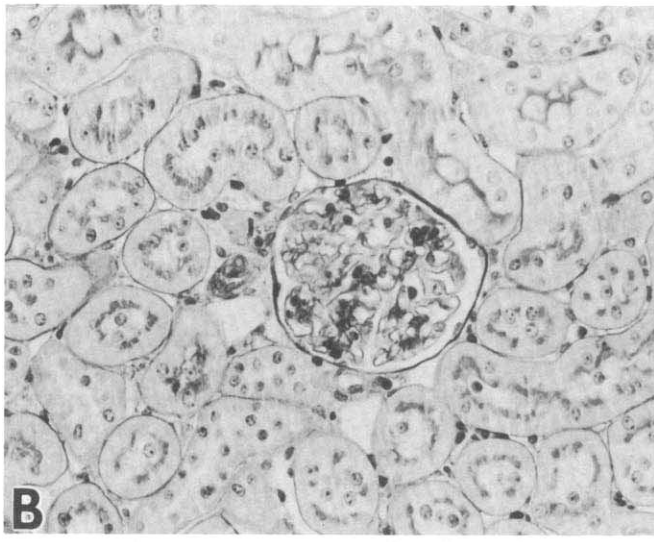
Renal histology

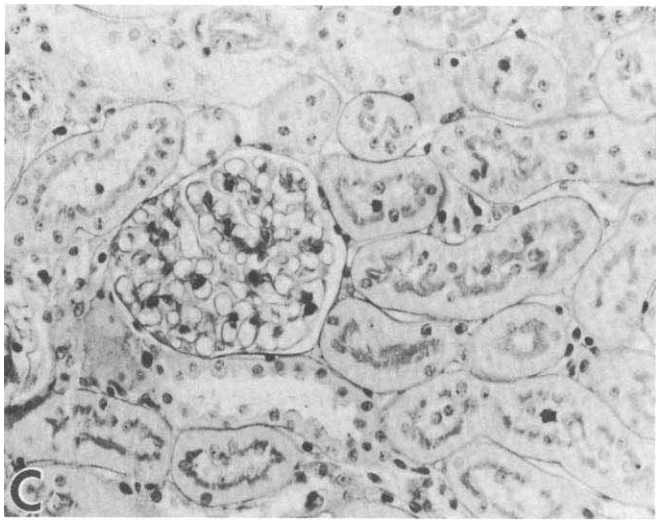
Renal morphology in the control, insulin-treated and untreated groups was normal by light microscopy 7 and 28 days after diluent or streptozotocin injection (Fig. 2). The vacuolization of renal tubular epithelium ascribed to streptozotocin induced nephrotoxicity [18] was not observed in either the untreated or the insulin treated group. There was a trend toward larger glomeruli in the DM and NPH groups compared to C both at 7 days (3.5 ± 0.3 and 3.8 ± 0.2 vs. 3.2 ± 0.2 × 10^5 μ^3, respectively), and at 28 days (C, 7.8 ± 0.3 and DM, 8.6 ± 0.3 × 10^5 μ^3), but these differences did not reach statistical significance.

In vitro hybridization of "medullary" RNA

In vitro mRNA-cDNA hybridization was performed on kidneys seven days post-injection to quantitate the procollagen α1(IV), α1(I) and β-actin mRNA levels in "medulla," comprised of the renal papillae, the inner and outer stripes of the medulla, and approximately 1 mm of the deep cortex, including a small number of juxtamedullary glomeruli. Mean procollagen α1(IV) mRNA levels in "medulla" were significantly higher in DM (244 ± 57%) than in C (100 ± 12%) and NPH (86 ± 11%) ($P < 0.01$, DM vs. C and DM vs. NPH; Fig. 3). Marginal increments in "medullary" procollagen α1(I) mRNA levels were observed in the rats with untreated diabetes compared to non-diabetic controls and insulin treated diabetic rats (C = 100 ± 13%; DM = 141 ± 5%; NPH = 120 ± 11%; ANOVA, not significant; Fig. 3). Changes in β-actin were also not significantly different (C = 100 ± 10%; DM = 164 ± 39%; NPH = 101







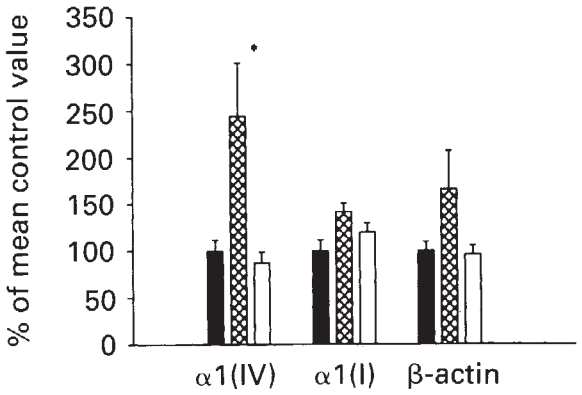


Fig. 3. *"Medullary" procollagen α1(IV), α1(I) and β-actin mRNA levels 7 days after diluent or streptozotocin injection.* The procollagen α1(IV) mRNA level is significantly higher in DM (▩) for both the DM vs. C (■) and DM vs. NPH (□) comparisons ($P < 0.01$).

± 10%; ANOVA not significant). Although numerical increases in mRNA levels were observed in all three mRNAs studied in untreated diabetic rats, the increment in procollagen α1(IV) mRNA was of the greatest magnitude and was the only comparison to achieve statistical significance by ANOVA and by the Student's *t*-test corrected for multiple comparisons. Thus, while all mRNAs studied were numerically increased, the magnitude and significance of the change was greatest for (and therefore relatively specific for) procollagen α1(IV).

There were weak but statistically significant relationships between "medullary" procollagen mRNA levels and serum glucose levels, proteinuria, glycosuria, and kidney weight corrected for body weight. Procollagen α1(IV) mRNA levels were significantly correlated to serum glucose levels ($r = 0.43$, $P < 0.05$) and proteinuria ($r = 0.43$, $P < 0.05$). Procollagen α1(I) mRNA levels correlated with glycosuria ($r = 0.38$, $P < 0.05$) and with kidney weight corrected for body weight ($r = 0.46$, $P < 0.05$). The relationship between procollagen α1(IV) mRNA levels and glycosuria was of a similar magnitude ($r = 0.37$) but fell just short of statistical significance ($P > 0.05$). No significant correlation was discerned between procollagen α1(IV) mRNA levels and kidney weight factored for body weight ($r = 0.29$, $P > 0.05$).

In vitro hybridization of glomerular RNA

In vitro mRNA-cDNA slot-blot hybridization was performed to quantitate the procollagen α1(IV), α1(I), α1(III) and β-actin mRNA levels in control, untreated diabetic and insulin treated diabetic rats seven days after diluent or streptozotocin injection. In addition, glomerular procollagen α1(IV) and β-actin mRNA levels were measured in control and untreated diabetic rats 28 days after injection. No significant increments were noted in procollagen α1(IV), α1(I), α1(III) or β-actin mRNA

Fig. 2. *Representative sections of deep renal cortex from rats 7 days after streptozotocin or buffer injections.* **A.** Control rat. There are no abnormalities of the tubules, interstitium or glomerulus. **B.** Diabetic rat with NPH insulin treatment. The tubulointerstitium and glomerulus are normal. **C.** Untreated diabetic rat. There is no vacuolization, necrosis or atrophy of tubules. The interstitium and glomerulus have no alterations. Periodic acid Schiff ×250.

Table 5. Glomerular procollagen and β-actin mRNA levels in control, NPH insulin-treated diabetic, and untreated diabetic rats expressed as a % of the mean control value

	C		NPH	DM	
	7 days	28 days	7 days	7 days	28 days
Procollagen α1(IV)	100 ± 22	100 ± 6	98 ± 12	69 ± 16	135 ± 33
Procollagen α1(I)	100 ± 24	—	115 ± 17	125 ± 15	—
Procollagen α1(III)	100 ± 16	—	—	82 ± 9	—
β-actin	100 ± 18	100 ± 19	85 ± 8	91 ± 1	140 ± 11

levels in the insulin treated or untreated diabetic rats compared to control animals seven days after injection (Table 5). For this reason, glomerular procollagen α1(IV) and β-actin mRNA levels were measured in control and untreated diabetic rats 28 days post-injection. Again, no significant differences between the two groups were observed in procollagen α1(IV) or β-actin mRNA levels (Table 5).

In situ hybridization

These autoradiographic studies were performed to localize the cellular origin of the increase in "medullary" procollagen α1(IV) mRNA levels measured in the untreated diabetic rat kidneys by in vitro hybridization. In situ hybridization with radiolabeled procollagen α1(IV) cDNA and antisense cRNA probes revealed similar findings.

Utilizing the cDNA probe, by light field microscopy, silver grains identifying the procollagen α1(IV) mRNA were rarely seen in tubules or glomeruli of control rats either seven days or longer than 120 days after diluent injection, consistent with a relatively low abundance baseline expression for this message (Fig. 4A). Renal tissue from insulin treated diabetic rats had few silver grains in proximal tubular cells or glomeruli, not appreciably increased in number from those in control kidneys (Fig. 4B). In contrast, rats made diabetic for seven days had clearly identifiable increased numbers of silver grains localized predominantly but not exclusively in proximal tubules of the deep cortex and outer medullary stripe (Fig. 4C). Fewer grains were present in proximal tubules of the outer cortex, as well as a scattering of grains in distal tubules and collecting ducts. The majority of tubules disclosing the increased number of silver grains were identified as proximal by their location, morphology and brush border staining with periodic acid Schiff. Silver grains on renal sections from rats with untreated diabetes for more than four months were distributed similarly to the rats with diabetes for seven days, but grains were present in greater number and tended to be more confluent (Fig. 4E). No increase in silver staining was present in glomeruli from animals diabetic for seven days, and basal expression of this mRNA as identified by this method was barely detectable.

By darkfield microscopy, the distribution of the staining was identical to that observed by light microscopy but was more apparent overall. In both control and NPH treated rats, rare white granules corresponding to sites of procollagen α1(IV) mRNA could be discerned in the renal tubules, predominating, but not exclusively in, the proximal tubules of the deep cortex and outer medullary stripe. Autofluorescence of tubular basement membranes was also apparent. In contrast, in untreated diabetic rats prominent and widespread proximal tubular staining was present in the deep cortex and outer medullary stripe, with scattered, less prominent granularity in the superficial cortex and inner medulla in untreated diabetic rats seven days after injection (Fig. 4D). This pattern was preserved in rats with untreated diabetes for more than four months, but, as in the light microscopic examination, heavier granularity was present in rats with more prolonged diabetes (Fig. 4F).

With the cRNA probe, the distribution of the increased procollagen α1(IV) mRNA in the diabetic rat kidney was virtually identical to that observed with the cDNA probe, these being predominant increments in proximal tubular cytoplasm, and, to a much lesser extent, increased expression in distal tubules. While the histologic distribution of the lesions were identical with the cDNA and cRNA probes, the cRNA tended to bind quantitatively more than the cDNA (Fig. 5A). Thus, the in situ studies were utilized only for the purpose of mRNA localization, and not for quantification.

No silver grains were present on sections hybridized with sense cRNA probes (Fig. 5B), or on those renal sections pre-treated with RNase prior to in situ hybridization.

Discussion

Thick glomerular and tubular basement membranes, expanded mesangial matrix, tubulointerstitial fibrosis and renal hypertrophy and hyperplasia are characteristics of the diabetic kidney. Since Type IV collagen is a constituent element of basement membranes and mesangial matrix, alterations in its synthesis and/or degradation may contribute to the development of diabetic renal disease. These experiments were performed in order to determine whether early diabetes is associated with an alteration in procollagen mRNA abundance. In this study, "medullary" and glomerular procollagen and β-actin mRNA levels were measured in rats 7 and/or 28 days after diluent or streptozotocin injection. In vitro hybridization experiments demonstrated a significant increase in procollagen α1(IV) mRNA levels in the "medullary" tissue of these diabetic rats, while procollagen α1(I) and β-actin mRNA levels were numerically but not statistically significantly elevated. Further studies utilizing in situ hybridization techniques localized the increase in procollagen α1(IV) mRNA predominantly to the proximal tubular cells of the deep cortex and outer stripe of the renal medulla. Glomerular procollagen α1(IV), α1(I), α1(III) and β-actin levels were not increased at the time points studied. Thus these experiments demonstrate an increase in proximal tubular procollagen α1(IV) mRNA levels in the kidneys of rats with untreated diabetes mellitus. This increment preceded any change in mRNA levels in the glomerulus. The specificity of this observation lies not so much in the uniqueness of the mRNA that is expressed, but rather in the restricted localization within the kidney of that enhanced expression.

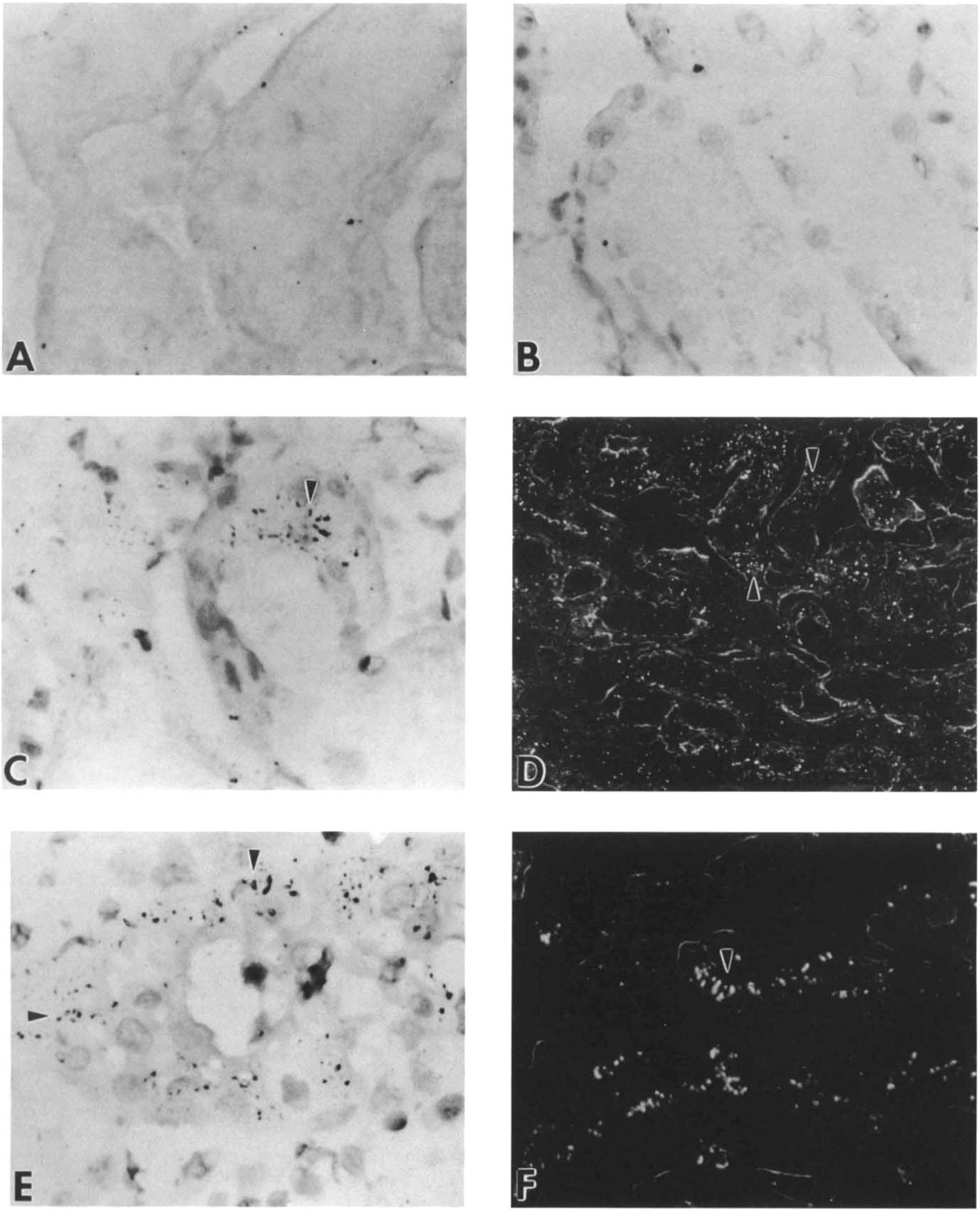


Fig. 4. *cDNA in situ hybridization for procollagen α1(IV) mRNA in proximal tubules of the deep cortex and outer medullary stripe.* **A.** Buffer injected control after 7 days. Few silver grains are evident. **B.** NPH insulin treated diabetes after 7 days. As in controls, few silver grains are present in proximal tubules. **C,D.** Untreated diabetes after 7 days. Moderate numbers of silver grains (arrowheads) are present within proximal tubular epithelium. **E,F.** Untreated diabetes after >4 months. Many silver grains (arrowheads) are within the proximal tubule cells. A,B,C,E-methyl green ×625. D,F Darkfield ×275.

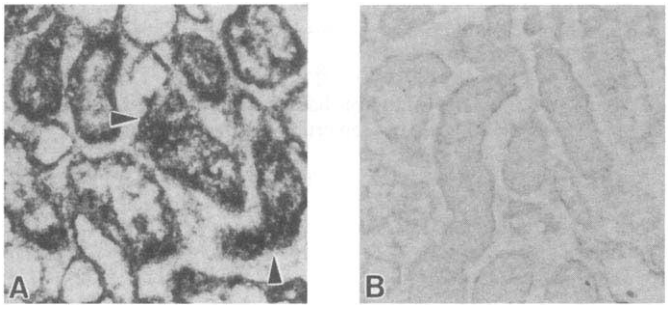


Fig. 5. *In situ hybridization of diabetic rat deep cortex using cRNA probe for procollagen α1(IV) mRNA.* **A.** Antisense probe. There is heavy labelling of proximal tubules with silver grains (arrowheads) with fewer grains in other tubules. **B.** Sense probe. There are virtually no silver grains present. Methyl green ×200.

The findings reported herein complement some and conflict with other work in experimental diabetes. In a model of non-insulin dependent diabetes in the KKAy mouse, Ledbetter et al demonstrated an increase in renal cortical procollagen α1(IV) mRNA levels beginning at two months of age, but becoming statistically significant at four months of age [44]. Studies to localize the cellular origin of this mRNA were not performed. Killen et al [45], in a preliminary study, reported a statistically significant increase in whole kidney procollagen α1(IV) and B1 laminin mRNA levels in insulin treated rats made diabetic by streptozotocin ($P < 0.05$) and a similar, but statistically insignificant trend for increased procollagen α1(IV) mRNA in untreated diabetic rats ($P > 0.05$). Our results differ from those of Killen et al in that the procollagen α1(IV) mRNA levels in our untreated diabetic rats were significantly ($P < 0.01$) higher than in our controls, and were normalized by insulin treatment. This discrepancy may be largely technical. The experiments by Killen et al were performed on RNA isolated from whole kidney, while ours were done on tissue comprised almost exclusively of medulla and renal papillae. Since the increase in message localizes to the deep cortex and the superficial medulla, it is possible that the failure to detect increases in procollagen α1(IV) mRNA levels in untreated diabetic rats is due to the presence of superficial renal cortex in the whole kidney study which "dilutes" the message, thereby diminishing the power to observe differences. While our study and the work of Ledbetter et al [44] and Killen et al [45] show an increase in procollagen α1(IV) mRNA levels in diabetic kidney under some experimental conditions, it should be noted that not all studies have been able to confirm a relationship between diabetes and increases in renal procollagen α1(IV) mRNA. Poulsom et al [46] showed diminished whole kidney procollagen α1(IV) mRNA levels in rats rendered diabetic by streptozotocin compared to controls. While it is difficult to speculate about the reasons for these discrepant findings, it is possible that these disparities may be ascribed to differences in rat strain, age, nutritional status, severity and/or duration of diabetes, method of RNA quantitation, cDNA probes utilized and, once again, segments of the kidney studied.

Taken together, however, these data tend to support the premise that the abnormal metabolic milieu of diabetes is capable of inducing increases in vivo in procollagen α1(IV) mRNA levels in the diabetic kidney. The precise mechanism for this event in the proximal tubule remains speculative. The effect of diabetes may be a direct one, mediated perhaps by an increase in glucose transport during states of hyperglycemia and marked glycosuria. In vitro, Ziyadeh has demonstrated that proximal tubules cultured in high glucose medium expressed increased levels of procollagen α1(I) and α1(IV) mRNA and secrete more Type I and Type IV collagen than tubules cultured in low glucose medium [47]. However, the observation in tubules in vitro lacks cell-type specificity. Similar increases in collagen production or mRNA levels have been noted in vitro when mesangial cells [48] umbilical vein endothelial cells [49], and neural connective tissue cells [50] are cultured in high glucose medium. Thus, the relative specificity of the localization in vivo suggests a mechanism other than simply high glucose concentrations, which might be predicted on the basis of these in vitro experiments to affect cell types besides tubules. One may speculate that the increased transport activity of the deeper proximal tubules during states of marked hyperglycemia are in some way linked to the induction of the collagen message. However, in this study, only a weak correlation was observed between the serum glucose levels and the procollagen α1(IV) mRNA levels, and no significant association was noted with the degree of glycosuria.

The relationship between diabetes and increased proximal tubular procollagen α1(IV) mRNA levels might also be a function of the development of renal hyperplasia or hypertrophy. However, in this study, while wet kidney weight corrected for body weights were similar in the insulin treated and untreated rats, elevations in procollagen α1(IV) mRNA occurred only in the latter group. This suggests that hypertrophy by itself is not a sufficient condition to induce measurable increments in procollagen α1(IV) mRNA levels. It is doubtful that increased renal function or hyperfiltration are responsible for the increments in the procollagen α1(IV) mRNA levels noted in these kidneys. This is borne out by the fact that the highest procollagen mRNA levels were seen in the group with the lowest creatinine clearance corrected for body weight (untreated diabetic group) and the lowest levels were seen in the group with the highest creatinine clearance corrected for body weight (insulin treated diabetic group). Finally, the increase in procollagen α1(IV) mRNA levels in the proximal tubules of diabetic rats may be due to streptozotocin nephrotoxicity. Increased proteinuria without concomitant albuminuria in our untreated diabetic rats compared to control rats at seven days but not 28 days suggests that there may have been tubular dysfunction in the diabetic rats sacrificed one week after injection. We believe that this tubular dysfunction was not due to streptozotocin nephrotoxicity, and that streptozotocin toxicity is not the cause of the increase in procollagen α1(IV) mRNA levels for the following reasons. The typical findings of vacuolated proximal tubular cells characteristic of rats with streptozotocin nephrotoxicity [51, 52] were not observed in our diabetic rats. In addition, rats receiving the same dose of streptozotocin, but who were treated with daily low dose insulin did not have abnormal proteinuria. Moreover, similar increases in presumptive tubular proteinuria occur in diabetic mice [53] where no streptozotocin is involved in lesion induction. Furthermore, by in situ hybridization, the proximal tubular expression of procollagen α1(IV) mRNA persisted in the rats injected with streptozotocin greater than four months previously, suggesting an

effect of prolonged duration. The persistence and possible enhancement of the mRNA signal in the tubules for a prolonged period of time after a single injection of streptozotocin argues against drug-induced nephrotoxicity as the inciting cause for the increase in mRNA observed. Thus, there is little likelihood that streptozotocin itself is directly related to increased message levels in this experiment.

While the mechanism of increased tubular procollagen α1(IV) mRNA levels remains uncertain in acute diabetes, it is interesting that low-dose insulin therapy insufficient to achieve euglycemia or to inhibit hypertrophy suppressed this rise. Experiments concerning the effect of insulin on collagen synthesis and accretion have been inconclusive. Insulin has been reported to increase glomerular collagen synthesis in vitro by some [54] and to decrease it by others [55, 56]. Low dose insulin treatment has previously been shown to inhibit the non-specific increase in total glomerular RNA observed in diabetic rats [57]. However, in our study, the increase in "medullary" mRNA levels in untreated compared to insulin treated rats was 2.8-fold for procollagen α1(IV) mRNA, 1.2 fold for procollagen α1(I) mRNA and 1.6-fold for β-actin mRNA. The greatest decrement in mRNA levels associated with insulin treatment was therefore observed for procollagen α1(IV). The disparity in the magnitude of the insulin effect on the mRNA levels for the different peptide chains studied suggests more specificity than merely a generalized suppression by insulin of all mRNAs expressed.

The molecular mechanisms underlying the increased procollagen α1(IV) mRNA levels in diabetic rat tubules cannot be discerned from this study. Enhanced gene transcription, as well as stabilization of the mRNA are both possible explanations for the greater abundance of procollagen α1(IV) mRNA observed in these experiments. The increase in proximal tubular procollagen α1(IV) mRNA levels was noted prior to the full expression of renal hypertrophy and before glomerulosclerosis and tubulointerstitial fibrosis developed in these diabetic animals. These findings support the premise that diabetic renal disease may be associated with an acquired dysregulation in the process of transcription and/or translation of basement membrane collagen.

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